学 位 論 文 の 要 旨

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学　位　論　文　名　The HSP70 and Autophagy Inhibitor Pifithrin-μ Enhances the Antitumor Effects of TRAIL on Human Pancreatic Cancer

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論 文 内 容 の 要 旨

INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in cancer cells with little cytotoxicity to normal cells. Therefore, TRAIL has been applied for several clinical trials. However, the binding of TRAIL to death receptors on cancer cells activates NF-κB, MAPK, and AKT signaling, thereby promoting cell proliferation and apoptosis-resistance.

Cancer cells acquire therapy-resistance through a variety of mechanisms. One is increased expression of heat shock protein (HSP)70. Importantly, increased expression of HSP70 in cancer cells has been reported to be associated with malignant features and poor prognosis in cancer patients. Alternatively, autophagy has received much attention as a mechanism of therapy-resistance in cancer cells. Its fundamental role in cells is cytoprotection under starvation and stress conditions, but this protective function can render cancer cells therapy-resistant. These lines of evidence suggest that HSP70 and autophagy are promising targets in cancer treatment.

Pifithrin (PFT)-μ (2-phenylethynesulfonamide) was initially identified as a small-molecule inhibitor of p53. Thereafter, PFT-μ was revealed to interact selectively with HSP70 and to inhibit its functions. Additionally, PFT-μ can inhibit autophagy, as well as the NF-κB pathway. In the current study, we investigated the antitumor effects induced by the combination of TRAIL and PFT-μ against human pancreatic cancer, which is highly resistant to conventional anti-cancer therapies.

MATERIALS AND METHODS

Four human pancreatic cancer cell lines (MiaPaca-2, Panc-1, AsPC-1, and BxPC-3) were used. Cell viability was evaluated using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay. Cell death was evaluated by flow cytometry after the Annexin V/PI staining. Cell cycle and proliferation of cancer cells were evaluated by the BrdU/7AAD Prolifesration Kit. Immunoblot was performed to examine the protein levels of LC3, cyclin D1, c-Myc, Beclin-1, Cathepsin L, and IκBα. To selectively knock down HSP70 or Beclin-1, HSP70 siRNA and Beclin-1 siRNA were transfected using Lipofectamine™ RNAiMAX, respectively, according to the manufacturer's instructions

Autophagy was evaluated by two different methods; expression of type-II LC3 by immunoblot and GFP-LC3 foci by confocal imaging. GFP-LC3-expressing cancer cells were prepared by transfction of the pcDNA3.1 vector that was inserted by *LC3B* (NM_022818) gene using Lipofectamine 2000. Confocal imaging was also used to examine the translocation of NF-κB to the nucleus after staining with anti-NF-κB p65 antibody, followed by Alexa Fluor 488-conjugated second antibody.

To examine the *in vivo* antitumor effect, BALB/c *nu/nu* mice were inoculated in the right flank with MiaPaca-2 cells with Matrigel at a volume ratio of 1:1. On day 20, the mice were pooled and divided into four groups, and started to inject with PFT-μ intraperitoneally and with TRAIL intratumorally. Thereafter, tumor size was measured twice weekly.

RESULTS AND DISCUSSION

Among four pancreatic cancer cell lines, MiaPaca-2, BxPC-3, and Panc-1 cells were highly, moderately, and lowly sensitive to TRAIL, respectively. In contrast, AsPC-1 cells were entirely resistant to TRAIL. In subsequent experiments, we focused on MiaPaca-2 and Panc-1 cells. We next tried to determine roles of HSP70 and autophagy when cancer cells were treated with TRAIL. Knockdown of HSP70 markedly increased the percentage of Annexin V^+ Panc-1, but not MiaPaca-2, cells upon TRAIL treatment. Alternatively, TRAIL treatment increased the expression of LC3-type II in MiaPaca-2 cells, while LC3-type II was expressed in Panc-1 cells without TRAIL treatment. Although GFP-LC3 foci were detected in GFP-LC3-transfected MiaPaca-2 and Panc-1 cells, even without TRAIL treatment, they appeared to increase in both number and size after TRAIL treatment. Knockdown of Beclin-1, a molecule required for autophagy, decreased the viabilities and increased the percentages of Annexin V^+ MiaPaca-2 and Panc-1 cells upon TRAIL treatment. These results indicate that both HSP70 and autophagy play protective roles in TRAIL-induced antitumor effects on pancreatic cancer cells.

We next determined whether TRAIL-induced antitumor effects were enhanced by PFT-μ. When suboptimal doses of TRAIL and PFT-μ were combined, additive effects were evident in three cell lines other than AsPC-1. Combining PFT-μ with TRAIL decreased the colony-forming ability and increased the number of Annexin V^+ cells of MiaPaca-2 and Panc-1 compared to those treated with either agent alone. We also investigated the underlying mechanisms of the combination therapy. TRAIL-induced increase of Annexin V^+ cells was completely inhibited by the pan-caspase inhibitor z-VAD, while z-VAD rescued in part PFT-μ–induced cell death. We further determined whether growth arrest was involved in the antitumor effects of PFT-μ by evaluating BrdU uptake and 7AAD staining and by examining the expression of proliferation-related proteins. These results indicate that PFT-μ induces two types of antitumor responses in pancreatic cancer cells; cell death, both caspase-dependent and -independent, and cell-growth arrest. Additionally, PFT-μ inhibited degradation of IκBα in TRAIL-treated cancer cells, implying that PFT-μ can antagonize TRAIL-associated NF-κB activation in pancreatic cancer cells.

Finally, we evaluated whether combination therapy with TRAIL plus PFT-μ exerted an antitumor effect against established human pancreatic cancer in a xenograft mouse model. Although the systemic administration of PFT-μ had no antitumor effect and local injections of TRAIL decreased tumor growth moderately, combination therapy with TRAIL plus PFT-μ significantly suppressed tumor growth.

Since pancreatic cancer is highly resistant to conventional anti-cancer therapies and is associated with a very poor prognosis, new treatment modalities to enhance the efficacy of current treatments are required. In this study, we investigated the possibility that PFT-μ, a small-molecule HSP70 inhibitor that also has the ability to alter autophagy, could enhance TRAIL-induced antitumor effects on human pancreatic cancer cells. As a result, we found that both HSP70 and autophagy are, at least in part, responsible for the TRAIL resistance of cancer cells, and that PFT-μ enhances TRAIL-induced antitumor effects on human pancreatic cancer cells.

CONCLUSION

PFT-μ has the potential to induce two types of antitumor effects, *i.e.*, cell death and cell-growth arrest, and enhances TRAIL-induced antitumor effects against human pancreatic cancer cells both *in vitro* and *in vivo*. Therefore, PFT-μ is a promising agent to enhance the antitumor effects of TRAIL or agonistic antibodies against death receptors.